Nitric oxide suppresses cerebral vasomotion by sGC-independent effects on ryanodine receptors and voltage-gated calcium channels

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Nitric oxide suppresses cerebral vasomotion by sGC-independent effects on RYRs and voltage-gated calcium channels

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Short title: sGC-independent effects of nitric oxide on cerebral vasomotion

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ABSTRACT

Background/Aims: In cerebral arteries, NO release plays a key role in suppressing vasomotion. Our aim was to establish the pathways affected by NO in rat middle cerebral arteries. ***Methods***: In isolated segments of artery, isometric tension and simultaneous measurements of either smooth muscle membrane potential or $[Ca^{2+}]_{SMC}$ changes were recorded. ***Results***: In the absence of L-NAME, asynchronous propagating Ca^{2+} waves were recorded that were sensitive to block with ryanodine, but not nifedipine. L-NAME stimulated pronounced vasomotion and synchronous Ca^{2+} oscillations with close temporal coupling between membrane potential, tone and $[Ca^{2+}]_{SMC}$. If nifedipine was applied together with L-NAME, $[Ca^{2+}]_{SMC}$ decreased and synchronous Ca^{2+} oscillations were lost, but asynchronous propagating Ca^{2+} waves persisted. Vasomotion was similarly evoked by either IbTx, or by ryanodine, and to a lesser extent by ODQ. Exogenous application of NONOate stimulated endothelium-independent hyperpolarization and relaxation of either L-NAME-induced or spontaneous arterial tone. NO-evoked hyperpolarization involved activation of BK_{Ca}-channels via RYRs, with little involvement of sGC. Further, in whole cell mode, NO inhibited current through L-type VGCC (I_{CaL}), which was independent of both voltage and sGC. ***Conclusion***: NO exerts sGC-independent actions at RYRs and at VGCC, both of which normally suppress cerebral artery myogenic tone.

Keywords: nitric oxide, membrane potential, calcium signaling, vascular smooth muscle, cerebral arteries, vasomotion

INTRODUCTION

Cerebral arteries typically display spontaneous, submaximal constriction that is dependent on the level of intraluminal pressure or isometric stretch, termed myogenic tone. This myogenic tone is an essential mechanism in the local control of blood flow and tissue perfusion in the cerebral vasculature both *in vivo* and *in vitro*, and in many other vascular beds [1,2]. The development of myogenic tone is generally characterised by vascular smooth muscle cell depolarization, leading to an increase in the intracellular [Ca^{2+}] ($[Ca^{2+}]_{SMC}$) and associated constriction of the artery [1,3]. Myogenic responses, by definition, can occur without a functional endothelial cell layer; however, the endothelium can considerably modulate the degree of myogenic tone by releasing a number of factors including nitric oxide (NO), prostacyclin and endothelium-derived hyperpolarizing factor.

In addition to suppressing myogenic tone, endothelium-derived factors also modulate the vasomotion that often occurs in tandem with the development of myogenic constriction. Vasomotion describes rhythmic oscillations in tension or diameter that are normally synchronous with oscillations in Ca^{2+} and membrane potential (E_m). In the brain, oscillations in middle cerebral artery blood flow velocity (as a result of vasomotion) have been observed in many species, including humans [4] and rats [5]. The role of the endothelium in the control of vasomotion is unclear; in some vascular beds the NO/cGMP pathway has been shown to augment vasomotion [6]. However in other beds, including the cerebral vasculature [5,7], NO/cGMP attenuates this response as NOS inhibitors stimulate vasomotion. This vasomotion manifests as a reduction in capillary blood flow, which tends to oscillate in synchrony within the bed [8]. Therefore any disruption of the ability to synthesize NO can potentially lead to vasomotion and/or spasm, as observed under pathophysiological conditions such as subarachnoid haemorrhage [9,10].

In arteries isolated from both coronary [11,12] and cerebral [13-20] beds, a continual, basal release of NO suppresses myogenic tone, with inhibition of NO synthase (NOS) leading to depolarization and constriction in the absence of vasoconstrictor agents. NO can either stimulate hyperpolarization and closure of voltage-gated Ca^{2+} channels (VGCC), or directly close VGCC, both of which suppress myogenic tone. In terms of hyperpolarization, NO can activate smooth muscle cell BK_{Ca}-channels either directly [21-23] or via PKG-dependent mechanisms [24,25]. NO can also stimulate ryanodine-sensitive calcium stores (by opening the ryanodine receptor, RYR) in the sarcoplasmic reticulum, evoking discrete calcium events termed 'sparks' that activate adjacent clusters of BK_{Ca}-channels. This mechanism has been suggested to underpin NO-dependent relaxation in the rat posterior cerebral artery [26] where the presence of NO is reported to be a prerequisite to activate the RYRs. Stimulation of RYRs by NO could be either direct or indirect, e.g. nitrosylation of thiol groups [27], or via cGMP mediated phosphorylation of the channel and the sarcoplasmic reticulum calcium ATPase [28], respectively. In addition, NO can close VGCC in a membrane potential-independent manner, which can occur either via sGC/PKG [29-31], and/or by nitrosylation [32-34].

Therefore we investigated further the mechanisms underlying the modulation of myogenic tone and the development of vasomotion associated with the basal release of NO in the rat (middle) cerebral arteries. Although our data support the suggestion that NO does stimulate RYR channels to release calcium that drives BK_{Ca}-channel mediated hyperpolarization, they also suggest two further important aspects of NO activity. First, that inhibition of NO synthase masks (rather than inhibits) spontaneous oscillations in smooth muscle cell calcium due to activation of VGCCs and the appearance of vasomotion, which is consistent with activation of RYRs via NO-independent pathways. Second, a direct inhibitory action of NO on VGCCs can suppress cerebral artery myogenic tone.

MATERIALS AND METHODS

Male Wistar rats (200-300 g) were euthanized using procedures defined by the Animals (Scientific Procedures) Act 1986, UK (Schedule 1 procedure) and the brain was rapidly removed and stored immediately in ice-cold physiological salt solution for a maximum of 30 min.

Simultaneous measurement of tension and membrane potential

A 2 mm segment of the middle cerebral artery (internal diameter of ~175 μm) was mounted in a Mulvany-Halpern myograph (model 410A, Danish Myotechnology) in Krebs solution containing (mM): NaCl, 118.0; $NaCO_3$, 25; KCl, 3.6; $MgSO_4 \cdot 7H_2O$, 1.2; KH_2PO_4, 1.2; glucose, 11.0; $CaCl_2$, 2.5; and gassed with 95 % O_2 and 5 % CO_2 at 37 ºC. The vessels were allowed to equilibrate for 20 min and were then tensioned to 1-1.5 mN (approximates wall tension at 60 mmHg). Vessel viability was assessed by the addition of exogenous K^+ (15-55 mM), only vessels developing tension ≥3 mN were used. Endothelial cell viability was assessed by the ability of SLIGRL (20 μM; a protease-activated receptor 2 ligand) to relax U46619 induced tone by >70 % and to hyperpolarize the smooth muscle cell membrane by >15 mV. All blocking drugs were allowed to equilibrate for 20 min before study except: nifedipine and ryanodine which produced immediate responses or whose effects were studied over a 20 min period. In some experiments, endothelial cells were removed by gently rubbing the luminal surface with a human hair; subsequent relaxation of <15 % to SLIGRL (20 μM) was considered as successful removal. Smooth muscle cell tension and E_m were measured simultaneously as previously described [35] and were recorded with the use of Powerlab system (AD instruments, Australia). Briefly,

individual smooth cells were impaled with a glass electrode (filled with 2 M KCl, tip resistance 60-100 MΩ) held perpendicular to the cells.

Simultaneous measurement of changes in $[Ca^{2+}]_{SMC}$ and tension

A segment of middle cerebral artery was mounted as described above except in a Mulvany-Halpern myograph designed for use on a confocal microscope (Model 120CW, Danish Myotechnology) and in MOPS buffer containing (mM): NaCl, 145; KCl, 4.7; $CaCl_2$, 2.0; $MgSO_4$, 1.17; MOPS, 2.0; NaH_2PO_4, 1.2; glucose, 5.0; pyruvate, 2.0; EDTA, 0.02; NaOH, 2.75 (the pH of the solution was adjusted to 7.39-7.41 at 37ºC using NaOH or HCl, as appropriate). The arteries were loaded with the calcium-sensitive fluorescent dye, Oregon Green 488 BAPTA-1 AM (10 μM; dissolved in DMSO and 0.02 % (w/v) Pluronic F-127) for 1 hour. After excitation at 488 nm, the fluorescence emission intensity at 515 nm was recorded using a spinning disc confocal microscope (Yokogawa CSU22, Japan) fitted with an Andor iXON DV887ECS-BV camera (Andor, UK) mounted on an Olympus IX70 inverted microscope (Olympus, Japan) using a water immersion objective (x40, aperture 0.8, working distance 3.3 mm, Olympus, Japan) and images (512 x 512 pixels, 20 Hz) stored for offline analysis (iQ, Andor). Following background subtraction, average, relative changes in $[Ca^{2+}]_{SMC}$ were calculated as changes in intensity of fluorescence divided by fluorescence at time = 0 s (F/F_0), within selected cell regions (5x5 pixels).

Isolated smooth muscle cell patch clamp experiments

Freshly dissected middle cerebral arteries were placed in ice-cold Ca^{2+}-free isolation solution containing (mM): NaCl, 140; KCl, 4.7; $MgCl_2$, 1.2; glucose, 10; and HEPES, 10 (pH 7.4). After

incubation on ice for 20 min, the arteries were transferred to Ca^{2+}-free isolation solution, containing 1 mg/ml albumin, 1mg/ml papain (Sigma), and 1 mg/ml dithiothreitol, and allowed to digest for 20 min at 37°C. The tissue was then transferred into a solution containing 0.1 mM $CaCl_2$ and 1 mg/ml collagenase type H (Roche) + 1 mg/ml collagenase type F (Sigma). Following digestion for 10 min at 37°C, the tissue was washed in isolation solution containing 1 mg/ml albumin and 0.1 mM $CaCl_2$. After gentle trituration, cells were centrifuged for 5 min at 1000 rpm, the supernantant removed, and resuspended in fresh isolation solution. The concentration of extracellular calcium was increased over the next 30 mins to 750 μM. Freshly isolated cells were maintained on ice for use on the same day.

Cells were placed in a heated recording chamber (RC-25F, Warner Instruments) and left for ~10 min to adhere to the cover glass. Cells were then continually superfused (~1 ml/min) with heated solution (SH-27B Inline Heater, Warner Instruments) via a multi-barrel gravity-fed perfusion system. Experiments were performed using an agar bridge (2% agar filled with 3 M KCl). During seal formation, cells were superfused with physiological saline solution (PSS) containing (mM): NaCl, 140; KCl, 4; $CaCl_2$, 1.5; $MgCl_2$, 1.2; HEPES, 10; glucose, 10; pH=7.4. To record membrane potential, the pipette solution contained (mM): KCl, 130; NaCl, 10; HEPES, 10; $MgCl_2$, 0.5; $CaCl_2$, 0.5; and Amphotericin B (200 μg/ml). To record L type calcium current (I_{CaL}), the whole cell mode was used and Ba^{2+} was used as the charge carrier. Cells were perfused with solution containing (mM): NaCl, 120; CsCl, 4; TEA-Cl, 10; $BaCl_2$, 10; $MgCl_2$, 1.2; HEPES, 10; glucose (pH=7.4). The pipette solution contained (mM): CsCl, 130; $MgCl_2$, 0.4; HEPES, 10; EGTA 2; $CaCl_2$, 0.4; GTP, 0.5; MgATP, 5; pH=7.3. The osmolarity of all solutions was measured and corrected to 300 ± 5 mOsm using mannitol. All electrophysiological recordings were performed at 37°C.

I_{CaL} was recorded using a 1 s ramp protocol, from –100 to +80 mV from a holding potential of –80 mV at a frequency of 0.05 Hz. Nifedipine (1 μM) was applied at the end of the protocol, and subtracted from the current records obtained in barium containing solution, and the data presented as nifedipine-sensitive current. Cell membrane capacitance was measured using a 10 mV hyperpolarizing step and used to correct I_{CaL} currents for cell size. Currents were expressed as current density (pA/pF). Any cell exhibiting current rundown in control conditions was excluded from the analysis. NONOate was freshly diluted with PSS, and infused via an injection port in the superfusion line directly upstream from the recording chamber. In experiments with the sGC inhibitor ODQ, cells were incubated in 10 μM ODQ for 15 mins, and it was also included in the perfusion solutions.

Data were analyzed and leak subtracted offline using pClamp 8 (Axon Instruments). Values are expressed as mean ± SEM of n cells (from at least 3 animals). The paired two-tail *t*-test was used to compare parameters obtained in control and test conditions in the same cell. A non-paired *t*-test was used to compare the differences between groups of data.

Solutions and drugs

Exogenous K^+ was added as an isotonic solution, and expressed as the final bath concentration. Caffeine, L-NAME (N^G-nitro-L-arginine methyl ester), nifedipine, ryanodine, BayK 8644 and papaverine were all obtained from Sigma (UK). IbTx was obtained from Latoxan (France); DEA-NONOate from Alexis (UK); ODQ (1H-(1,2,4)oxadiazolo(4,3-a)quinoxalin-1-one) from Tocris (UK); SLIGRL from Auspep (Australia); Oregon Green 488 BAPTA-AM from Molecular Probes (UK); TRAM-34 was a gift from Dr H. Wulff (University of California, Davis); and U46619 was from Calbiochem (UK). All drugs were made in 0.9 % NaCl except ryanodine, nifedipine, ODQ, U46619

and Amphortericin B in DMSO; NONOate in 0.01 M NaOH (and stored at -80 ºC); and (-) BayK 8644 in EtOH. All subsequent dilutions of all drugs were made in 0.9 % NaCl and vehicle had no effect. NONOate dilutions were kept on ice in the dark and were discarded after 20 min.

Statistical analysis

Results are expressed as the mean ± SEM of *n* animals. Relaxation is expressed as the peak percentage reduction of the total vascular tone (from the myogenic tone to the tension/diameter following addition of papaverine, 150 μM) or as mN, as appropriate. Constriction is expressed in mN or as a percentage of maximal constriction induced by exogenous K^+ (55 mM), as appropriate, all values were the peak values. When oscillations in membrane potential or tension were observed, values are the average of 10 s. Graphs were drawn and statistical comparisons made using either Student's *t*-test, or one-way ANOVA with Tukeys or Dunnetts post-hoc test using Prism software (Graphpad, USA).

RESULTS

Effect of inhibiting NO synthase, sGC, RYRs and BK_{Ca}-channels on myogenic tone

Rat middle cerebral arteries exhibit myogenic tone in a wire myograph equivalent to ~15 % of the maximum tension the vessel can develop [18], and associated with a resting membrane potential (E_m) of -50 ± 0.2 mV (*n* = 9). Addition of the NO synthase inhibitor, L-NAME (100 μM) evoked depolarization (to E_m -43.7 ± 1.9 mV, *n* = 6) and constriction (increase in tension of 3.7 ± 0.5 mN, *n* =

7; Figure 1). In addition, oscillations in E_m developed, temporally linked to oscillations in tension (Figure 1, Table 1). Fluorescence imaging revealed that in unstimulated control arteries, smooth muscle cells displayed spontaneous and asynchronous propagating Ca^{2+} waves (172 of 210 cells, Figure 1, online Movie 1). The oscillations occurred with a frequency of 0.27 ± 0.02 Hz (*n* = 21, Table 1) and were not associated with any change in tension (Figure 1). Addition of L-NAME increased the global $[Ca^{2+}]_{SMC}$ (data not shown) associated with the development of synchronous Ca^{2+} oscillations between smooth muscle cells that clearly linked temporally to changes in tension (Figure 1, online Movie 2, Table 1).

The BK_{Ca}-channel inhibitor iberiotoxin (IbTx, 100 nM) and the sGC inhibitor ODQ (10 μM) both mimicked this effect of L-NAME. Each caused depolarization (to E_m -40.3 ± 1.8 and -35.5 ± 6.0 mV *n* = 5 and *n* = 3, respectively; Figure 2) and vasoconstriction (increases in tension of 3.7 ± 0.8 and 3.8 ± 0.7 mN *n* = 6 and *n* = 3, respectively; Figure 2) associated with the development of oscillations in E_m temporally linked to oscillations in tone (Table 1). Note that the vasomotion induced by ODQ was at a significantly lower frequency than that with either IbTx or L-NAME (Table 1).

Inhibition of RYRs with ryanodine (10 μM) also mimicked the effect of L-NAME causing depolarization (13.5 ± 3.6 mV) and tension increases (2.3 ± 0.2 mN, *n* = 5), and associated development of synchronous E_m oscillations temporally linked with tension oscillations (Figure 3, Table 1). Similarly, ryanodine stimulated synchronous Ca^{2+} oscillations in phase with tension changes (Figure 3, Table 1).

Effect of L-NAME and ryanodine on oscillations in $[Ca^{2+}]_{SMC}$ in the presence of nifedipine

As ryanodine and L-NAME each evoke constrictor responses associated with depolarization (and consequent calcium entry via VGCC) the effects of these drugs were assessed in the presence of the L-type VGCC inhibitor, nifedipine (1 μM). Under control conditions, nifedipine alone hyperpolarized (6.4 ± 2.4 mV) and relaxed (0.76 ± 0.03 mN) myogenic tone ($n = 4$), associated with a slight but significant reduction in both the frequency (0.20 ± 0.02 Hz, $n = 4$, $P < 0.01$) and the number of cells exhibiting asynchronous propagating Ca^{2+} waves (to 70 %, 28 of 40 cells; Figure 4). Subsequent addition of L-NAME repolarized the smooth muscle E_m (depolarization of 7.5 ± 3.8 mV, $n = 4$) and caused a small increase in tension (0.8 ± 0.01 mN, $n = 4$), returning E_m and tension values close to values recorded in quiescent vessels. In the presence of nifedipine, L-NAME had no significant effect on the number of cells exhibiting asynchronous propagating Ca^{2+} waves (68 %, 27 of 40 cells) or the wave frequency (0.22 ± 0.02 Hz, $n = 4$; Figure 4). In contrast, ryanodine completely abolished these Ca^{2+} waves (to 0 in 60 cells; Figure 4).

Effect of blocking VGCC and application of exogenous NO or caffeine on L-NAME-induced tone

In vessels pre-constricted with L-NAME, nifedipine (1 μM) abolished oscillations in E_m and caused a repolarization (hyperpolarization of 11.7 ± 1.9 mV, $n = 3$) to circa the resting membrane potential in the absence of NOS inhibition. This was associated with complete reversal of L-NAME induced tone (93.1 ± 1.6 %, $n = 3$). Furthermore, nifedipine caused a large decrease in $[Ca^{2+}]_{SMC}$ (data not shown) and abolished the synchronous Ca^{2+} oscillations between SMC, unmasking the asynchronous propagating Ca^{2+} waves (compare Figure 4B to Figure 1B).

In vessels pre-constricted with L-NAME, application of caffeine (30 μM – 3 mM) induced concentration-dependent hyperpolarization and relaxation (log EC_{50}: -3.43 ± 0.10; 3 mM: 16.4 ± 5.0 mV and 91.8 ± 4.2 % relaxation, *n* = 8, Figure 5A). Hyperpolarization and relaxation to caffeine (1 mM: 16.5 ± 2.0 mV and 82.6 ± 3.7 %, respectively, *n* = 6) were attenuated by IbTx (Figure 5A) and by ryanodine (1 mM: 4.4 ± 1.9 mV and 40.0 ± 30.9 %, respectively, *n* = 6).

Application of the NO donor NONOate (3 nM – 1 μM) stimulated concentration dependent hyperpolarization and relaxation (log EC_{50}: -7.48 ± 0.05; 1 μM: 13.3 ± 2.1 mV and 83.0 ± 2.5 % relaxation, *n* = 5, Figure 5B). The sGC inhibitor ODQ (1 μM) did not affect hyperpolarization to NONOate but significantly attenuated the relaxation (Figure 5B). Blockade of BK_{Ca}-channels with IbTx (100 nM) significantly inhibited both NONOate-induced hyperpolarization and relaxation (Figure 5B).

Effect of removing the endothelium on myogenic tone and the response to application of exogenous NO

Following removal of the endothelium, cerebral artery smooth muscle cells were depolarized (E_m -45.9 ± 2.2 mV, *n* = 12) and spontaneously developed tension (1.5 ± 0.2 mN, *n* = 12) sometimes (9 of 12 records) associated with oscillations in both E_m (amplitude: 4.5 ± 1.2 mV; frequency: 0.84 ± 0.20 Hz, *n* = 12) and tension. In these denuded cerebral arteries, L-NAME did not further increase tension (data not shown).

The NO donor, NONOate (3 nM – 1 μM) evoked concentration dependent hyperpolarization and relaxation in denuded arteries ($logEC_{50}$: -7.50 ± 0.06; 1 μM: -9.2 ± 2.2 mV and 78.0 ± 7.9 % relaxation, *n* = 6, Figure 5C). Ryanodine (10 μM) caused a small increase in tone, which was associated with slight depolarization (E_m -41.7 ± 1.0 mV, *n* = 12), and a significant increase in the amplitude of oscillations in E_m (23.3 ± 2.7 mV; frequency: 1.24 ± 0.09 Hz, *n* = 12, Figure 5C). These oscillations were not coupled to a detectable tension change. Ryanodine markedly reduced the hyperpolarization produced by NONOate but did not significantly affect the relaxation. Interestingly, NONOate reduced the amplitude and frequency of ryanodine-mediated oscillations in E_m (1 μM: Figure 5C). The addition of IbTx did not modify the effects of ryanodine, apart from further increasing the amplitude of oscillations by around 10 mV (amplitude significantly increased to 34.9 ± 3.4 mV, frequency 1.29 ± 0.09 Hz, *n* = 8, Figure 5C).

To further characterize the action of NO, experiments were performed to assess an action at VGCC. In endothelium-denuded arteries, IbTx had no significant effect on hyperpolarization and relaxation responses to NONOate (compare Figure 5C to Figure 6B). However in the additional presence of ODQ, the hyperpolarization and relaxation to NONOate were reduced (Figure 6A, 6B). Subsequent addition of the L-type VGCC opener BayK 8644 did not significantly alter membrane potential (hyperpolarization of 3.7 ± 3.8 mV) but contracted arteries (1.2 ± 0.2 mN, *n* = 5) and significantly increased both the frequency and amplitude of oscillations in E_m (Figure 6C). In the presence of this combination of inhibitors, the hyperpolarization to NONOate was effectively abolished, and the relaxation to NONOate markedly reduced.

Direct action of NO on VGCC

Isolated cells. The average resting E_m of isolated smooth muscle cells was -51.1 ± 2.0 mV (*n* = 12). In these unstretched and unstimulated cells, E_m tended to oscillate (amplitude of 15.4 ± 2.8 mV, *n* = 12), but a clear pattern was not observed (Figure 7A). In contrast, under similar conditions at 37ºC, the resting E_m of smooth muscle cells isolated from mesenteric arteries tended to remain stable at -54.5 ± 0.6 mV (with less frequent and lower amplitude oscillations of 5.2 ± 0.5 mV, *n* = 11). Addition of 1 μM NONOate to the superfusion solution stimulated hyperpolarization and abolished the oscillations in E_m (Figure 7A). In whole cell mode, steady state I_{CaL} was recorded for 1 min using the ramp protocol. Application of NONOate (1 μM) to the bath induced a significant reduction in I_{CaL} that was not inhibited by ODQ (Figure 7C, D, *n* = 6-7). The effect of NONOate on I_{CaL} was time-dependent (Figure 7D), so values were taken at 10 min following application of NONOate.

DISCUSSION

These data from the rat middle cerebral artery indicate that myogenic tone and vasomotion are normally suppressed by basal release of endothelium-derived NO that inhibits VGCC largely via sGC-independent pathways. This can occur either through an effect at RYR and activation of smooth muscle cell BK_{Ca}-channels, or a direct action independent of voltage. The activation of BK_{Ca}-channels appears to involve in part an indirect action of NO due to stimulation of Ca^{2+} release from ryanodine-sensitive Ca^{2+} stores but also in part a direct action of NO on the K_{Ca}-channel. Therefore upon inhibition of NOS, smooth muscle cell depolarization due to closure of BK_{Ca}-channels and the removal of an

inhibitory influence, both lead to opening of VGCCs which is followed by a rise in $[Ca^{2+}]_{SMC}$ and tension leading to arterial vasomotion.

The finding that myogenic tone is normally suppressed by basal release of NO in rat middle cerebral arteries is consistent with previous studies using cerebral arteries [14-19] and a variety of other vessels that exhibit myogenic tone including small coronary arteries [11,12]. By suppressing myogenic tone, NO also suppresses vasomotion in the middle cerebral artery. Nifedipine fully reversed the effects of L-NAME, reversing tension and abolishing synchronised oscillations in both E_m and $[Ca^{2+}]_{SMC}$. Therefore it is apparent that opening VGCCs is essential for vasomotion to develop, consistent with many other vessels [1,36]. Despite this, we cannot rule out the involvement of ion other channels. Once the intracellular Ca^{2+} levels rise and the membrane depolarizes, other channels would be stimulated to open, including voltage-gated Na^+-channels, Ca^{2+}-activated Cl^--channels, and K_{Ca}-channels. Furthermore, as both the endothelial and smooth muscle cells are coupled by homocellular and heterocellular gap junctions in this artery [37], it remains possible that the endothelium influences membrane potential through NO or other mediators. For example, changes in endothelial cell Ca^{2+} are responsible for the release of NO, so endothelial cell K_{Ca}-channels may also play a role in the observed changes in membrane potential.

While basal release of NO is known to suppress myogenic tone (and vasomotion), the precise mechanisms are unclear. However, it is likely that NO acts via multiple mechanisms, a few of which are shown in Figure 8. NO can suppress the contractile apparatus of the smooth muscle cells via the cGMP pathway. Indeed, ODQ produced increases in tension and depolarization (similar to L-NAME, albeit with a lower frequency of vasomotion), suggesting that sGC somehow stimulates hyperpolarization, perhaps via an action at BK_{Ca}-channels through PKG-dependent mechanisms

[24,25] or by an action on RYRs [27,28]. In addition, our data are consistent with a cGMP-independent action of NO at BK_{Ca}-channels, because BK_{Ca}-channel-mediated hyperpolarization induced by the NO donor, NONOate, was not significantly attenuated by ODQ. This suggests that endogenous NO activates BK_{Ca}-channels either directly [21,22] or via stimulation of Ca^{2+} release (e.g. sparks) from ryanodine sensitive stores (by opening RYRs). Evidence for the latter comes from the ability of ryanodine to block NONOate induced hyperpolarization in endothelium-damaged vessels. Despite this block, NONOate was still able to reduce the frequency and amplitude of the depolarizing spikes (oscillations in E_m) linked with the vasomotion generated by ryanodine. This suggests that NO acts to prevent the opening of the ion channel responsible for the depolarization. Further evidence consistent with a cGMP-independent action of NO on RYRs, was the ability of ryanodine to (a) stimulate vasomotion, mimicking the effect of NOS inhibition; and (b) inhibit the IbTx-sensitive hyperpolarization to caffeine.

Although it is likely that a major component of NO-induced suppression of myogenic tone involves a stimulation of Ca^{2+} release events, our data argue against an essential role for NO in the activation of RYRs. In the presence of nifedipine, L-NAME did not markedly prevent the basal asynchronous propagating Ca^{2+} waves, whereas ryanodine did. This is in contrast to previous observations in cerebral arteries by Mandala *et al* [26], who suggested that NO was absolutely essential for RYR activation (and thus for activation of BK_{Ca}-channels) because spontaneous Ca^{2+} sparks were reduced by around 50 % with NOS inhibitors or endothelium removal. However, following on from our observations it is likely that asynchronous propagating Ca^{2+} waves were masked by the Ca^{2+} influx through the L-type VGCCs and development of synchronous Ca^{2+} oscillations, as observed in the present study. Therefore, while the activation of BK_{Ca}-channels by NO likely involves direct stimulation of RYR-controlled Ca^{2+} stores, this action of NO is not an essential step in the activation of RYR. It follows that as RYR

stimulation is not *necessarily* associated with NO, an as yet unidentified process may also modulate vasomotion. In support of this conclusion, inhibition of RYR in the absence of a functional endothelium (and therefore NO synthesis) resulted in a small increase in tension as well as development of large, regular depolarizing oscillations in E_m.

Further experiments in the absence of functional endothelium showed that NONOate appears to directly inhibit VGCCs. In the presence of both IbTx and ODQ, NONOate responses mimicked those of nifedipine under control conditions and in the presence of L-NAME, that is, complete block of the oscillations in E_m associated with a small hyperpolarization, and relaxation. This direct effect of NONOate on VGCC was confirmed in isolated smooth muscle cells, where I_{CaL} was markedly reduced. The effect of NONOate on I_{CaL} in the isolated cells appears to be at least in part via a direct action on the channel protein or associated proteins, rather than via a cGMP-dependent mechanism. This is consistent with previous findings in the carotid body, where Summers et al. (1999) showed that NO-mediated inhibition of I_{CaL} occurs via S-nitrosylation of the channel protein, and that S-alkylation of the free cysteine residues by NEM prevented the modulation by the NO donor sodium nitroprusside, rather than via the activation of sGC.

Further evidence for the action of NO on I_{CaL} in our studies to be induced by nitrosylation rather than via the cGMP/PKG pathway may come from the time-course of NONOate induced inhibition, which took minutes to tens of minutes to occur. Previous studies of neuronal BK_{Ca} channels suggest that not only does nitrosylation require a higher concentration of NO than the PKG pathway, but it develops with a much slower time-course [38,39]. Further, transient receptor potential (TRP) channels can be activated by NO donors, TRPC6 channels being PKG-dependent, whereas TRPC5 channels are more slowly activated via S-nitrosylation [40].

The action of NO at VGCC was fully reversed by adding the direct opener of L-type VGCC, suggesting the sites of action are independent. Although there is evidence that both NO and BayK 8644 each evoke their effects on the L-type VGCC via the pore-forming α_{1c}-subunit, BayK 8644, which competitively competes with nifedipine, binds from the extracellular surface to access the dihydopyridine receptor site within the channel [41,42]. The site of NO-induced VGCC modulation by nitrosylation still remains to be elucidated. However, studies on other ion channels and transporters indicate that S-nitrosylation sites are primarily hydrophobic intracellular cysteine residues, flanked by positively charged basic residues [43]. In the skeletal muscle ryanodine receptor, the NO nitrosylation site has been identified as a hydrophobic cysteine residue at position 3635 of the calmodulin-binding domain [44]. Indeed, there is also evidence that VGCC function can be impaired by nitrosylation of an intracellular tyrosine residue (Y2134) situated in the src kinase protein binding domain of the carboxy terminal of the α_{1c}-subunit [45].

In summary, in rat middle cerebral arteries a basal release of NO from the endothelium suppresses myogenic tone. This suppression of myogenic tone is due, at least in part, to the ability of NO to stimulate BK_{Ca}-channels by activating ryanodine-sensitive Ca^{2+} stores. Following inhibition of NOS, the BK_{Ca}-channels close leading to depolarization, with an associated increase in tension and the development of vasomotion. Therefore, our data indicate that basal NO-release represents an important controlling mechanism on myogenic tone in cerebral arteries. In disease states where NO synthesis is compromised, disruption of this constitutive suppression of myogenic tone would be predicted to increase significantly the risk of brain ischaemia.

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Table 1. Amplitude and frequency of smooth muscle cell E_m, tension and synchronous Ca^{2+} oscillations.

	SMC E_m				**$[Ca^{2+}]_{SMC}$**	
	Oscillation amplitude		Oscillation frequency		Oscillation frequency	
	E_m (mV)	Tension (mN)	E_m (Hz)	Tension (Hz)	Ca^{2+} (Hz)	Tension (Hz)
L-NAME	16.4 ± 1.8	0.14 ± 0.2	0.85 ± 0.06	0.84 ± 0.05	0.75 ± 0.05	0.76 ± 0.04
IbTx	19.7 ± 2.3	0.11 ± 0.02	0.97 ± 0.05	0.96 ± 0.06	ND	ND
ODQ	8.1 ± 0.7*	0.13 ± 0.02	0.57 ± 0.06*	0.56 ± 0.07*	ND	ND
Ryanodine	19.5 ± 3.4	0.06 ± 0.01*	1.08 ± 0.06	1.06 ± 0.06	1.04 ± 0.04*	1.01 ± 0.05*

Data expressed as mean ± SEM, n = 4-11. Time-matched, paired values obtained from simultaneous records of either E_m and tension or Ca^{2+} and tension. *P<0.05 significant difference from L-NAME.

Figure 1. Spontaneous nitric oxide release prevents vasomotion. Original traces showing (A) simultaneous recordings of membrane potential (upper panels) and tension (lower panels) or (B) simultaneous recordings of $[Ca^{2+}]_{SMC}$ (upper 2 sets of panels) and tension (lower panels) under control resting conditions (left hand panels) or in the presence of the NO synthase inhibitor, L-NAME (100 μM; right hand panels), in rat middle cerebral arteries. Under control conditions, membrane potential and tension are relatively stable, and at the same time, $[Ca^{2+}]_{SMC}$ is constantly oscillating but these oscillations are asynchronous between smooth muscle cells and can be observed as waves passing along cells (asynchronous propagating Ca^{2+} waves). In the presence of L-NAME, the smooth muscle cells depolarized and developed regular depolarizing oscillations, which were associated with increased tension and oscillations in tension; the peaks in E_m immediately preceded peaks in tension. In the presence of L-NAME oscillations in $[Ca^{2+}]_{SMC}$ were now synchronized and regular (synchronous Ca^{2+} oscillations) and were temporally linked to oscillations in tension. The top coloured traces correspond to the average F/F_0 in 3 cells indicated by filled coloured squares on the images of the preparations (C), and the black traces are the average change in fluorescence from 10 equivalent regions in separate cells. The lower coloured traces correspond to the percentage maximum change in fluorescence in single cells indicated by the open coloured squares in (C). Bar = 20 μm. Movie files corresponding to the cropped regions shown in Control and L-NAME (dashed lines) are available online. Summary data are shown in Table 1.

Figure 2. Spontaneous activation of BK_{Ca}-channels and sGC prevent vasomotion. Original traces showing the effect of either (A) the BK_{Ca}-channel inhibitor, IbTx (100 nM) or (B) the sGC cyclase inhibitor, ODQ (10 μM) on simultaneous recordings of membrane potential (upper panels) and tension (lower panels). Both IbTx and ODQ caused depolarization and increased tension and a development of vasomotion.

Figure 3. Spontaneous activation of RYRs prevents vasomotion. Original traces of (A) simultaneous recordings of membrane potential and tension or (B) simultaneous recordings of $[Ca^{2+}]_{SMC}$ and tension. The traces show recordings obtained in the presence of the inhibitor of RYRs (ryanodine, 10 μM). Ryanodine caused depolarization and increased tension of the middle cerebral artery associated with development of depolarizing oscillations in E_m that were temporally coupled to changes in tension. Ryanodine also caused development of synchronous Ca^{2+} oscillations that were temporally linked to oscillations in tension. $[Ca^{2+}]_{SMC}$ responses from 3 randomly selected cells are displayed (colour) as well as the 10 cell average (black).

Figure 4. Spontaneous nitric oxide release does not inhibit control, asynchronous propagating Ca^{2+} waves. Original traces showing the basal, asynchronous propagating Ca^{2+} waves from 3 representative cells (colour) and the 10 cell average (black; upper traces) and associated tension records (lower traces) in rat middle cerebral arteries (A) in the presence of the L-type VGCC inhibitor, nifedipine (1 μM) and (B) the combination of nifedipine and the NO synthase inhibitor, L-NAME (100 μM). Under control conditions changes in $[Ca^{2+}]_{SMC}$ were not synchronized between individual cells and were not coupled to changes in tension (as in Figure 1A). Nifedipine had no effect on the size of the asynchronous propagating Ca^{2+} waves. Subsequent addition of L-NAME also had no effect on these Ca^{2+} waves. Average data are shown in (C) showing the frequency of Ca^{2+} waves and the percentage of cells exhibiting this behavior (left panel) and the associated tension (right panel) in control vessels and in the presence of nifedipine, nifedipine + L-NAME and nifedipine + ryanodine (10 μM). Ryanodine completely abolished the Ca^{2+} waves in all cells of all vessels tested (60 cells). Data expressed as means ± SEM.

Figure 5. Caffeine and NONOate stimulate hyperpolarization and relaxation. Concentration response curves showing hyperpolarization (left panels) and relaxation (right panels) produced by caffeine (A) or the NO donor DEA-NONOate (B) in endothelium-intact (+EC) or endothelium-damaged arteries (-EC, C). Vessels were pre-incubated with L-NAME (100 μM), IbTx (100 nM), ODQ (10 μM) and/or ryanodine (10 μM). Data are expressed as mean ± SEM, n = 4-9. * Significant difference from L-NAME (+EC) or control (-EC), $P < 0.05$; † Significant difference from Baseline, $P < 0.05$.

Figure 6. Application of exogenous NO (NONOate 3 nM – 3 μM) appears to directly inactivate VGCCs in endothelium-denuded middle cerebral arteries. Original trace (A) showing that in the combined inhibition of BK_{Ca}-channels (IbTx, 100 nM) and sGC (ODQ, 10 μM), NONOate induces a reduction in membrane potential oscillation frequency and amplitude (upper trace) that is associated with relaxation (lower trace). The effects of NONOate were fully reversed by an opener of L-type VGCCs (BayK 8644, 1 μM). Highlighted regions (gray lines) are reproduced in an extended time base to demonstrating that BayK 8644 fully reverses the effects of NONOate. Also shown are concentration response curves (B) showing the effect of NONOate on membrane potential and tension, as well as histograms (C) that show the effect of NONOate on oscillation frequency and amplitude in the presence of IbTx, the combined presence of IbTx and ODQ and in the additional presence of BayK 8644. Note that following inhibition of BK_{Ca} and sGC, NONOate-mediated relaxation does not seem to involve a true hyperpolarization but results from a reduction in both frequency and amplitude of the oscillations in membrane potential. Data are expressed as mean ± SEM, n = 4-5. *P<0.05 significant difference from control. †P<0.05 significant difference from baseline.

Figure 7. NONOate inhibits VGCC via a sGC-independent mechanism. In isolated smooth muscle cells at 37ºC under current-clamp conditions (A) the resting E_m oscillated. Addition of 1 μM NONOate

(indicated by arrow) hyperpolarized the cell and abolished the oscillations in E_m. (B) The voltage protocol for detecting I_{CaL} (top) resulted in inward current that was reduced by 1 μM NONOate (bottom). (C) Mean current voltage relationships under both control conditions (n = 6, left) and after pretreatment with ODQ (n = 7, right) show that the inhibition of I_{CaL} by 1 μM NONOate was not sensitive to ODQ, and (D) the peak current was reduced by approximately 50% under both conditions. Data are expressed as mean ± SEM. *P<0.05 significant difference from control. Panel E shows the effect of 1 μM NONOate (added at arrow) on peak I_{CaL} amplitude over time, for data shown in panels C and D. NONOate-induced I_{CaL} inhibition took minutes to occur, and was not due to current rundown (Time control).

Figure 8. Schematic depicting actions of NO in cerebral artery smooth muscle cells. Release of NO from endothelial cells can suppress vasomotion via multiple mechanisms. (i) Stimulation of sGC can relax smooth muscle cells via voltage-independent pathways. (ii) NO can directly activate BK_{Ca}-channels, leading to hyperpolarization, closure of VGCC and relaxation. (iii) The action of NO on BK_{Ca}-channel activity can be indirect, via a direct action of NO at RYRs, or (iv) via an intermediate (e.g. sGC/PKG). RyRs are also activated by NO-independent mechanisms (v) including those related to store filling via the Ca^{2+}-ATPase (SERCA). This depiction is based on the close association of RyRs to BK_{Ca}-channels, which are spatially separated from the Ca^{2+} release and influx mechanisms associated with contraction [46,47].

Online supplementary material

The online supplementary material shows two movies of the Ca^{2+} events in middle cerebral arteries mounted in a wire myograph. Under control conditions (Movie 1) spontaneous, asynchronous propagating Ca^{2+} waves can be observed in the individual smooth muscle cells. After the addition of L-NAME, the artery contracted and developed vasomotion. Under these conditions, the synchronous Ca^{2+} oscillations were observed (Movie 2). See Figure 1 for traces of tension and $[Ca^{2+}]_{SMC}$.

Figure 1

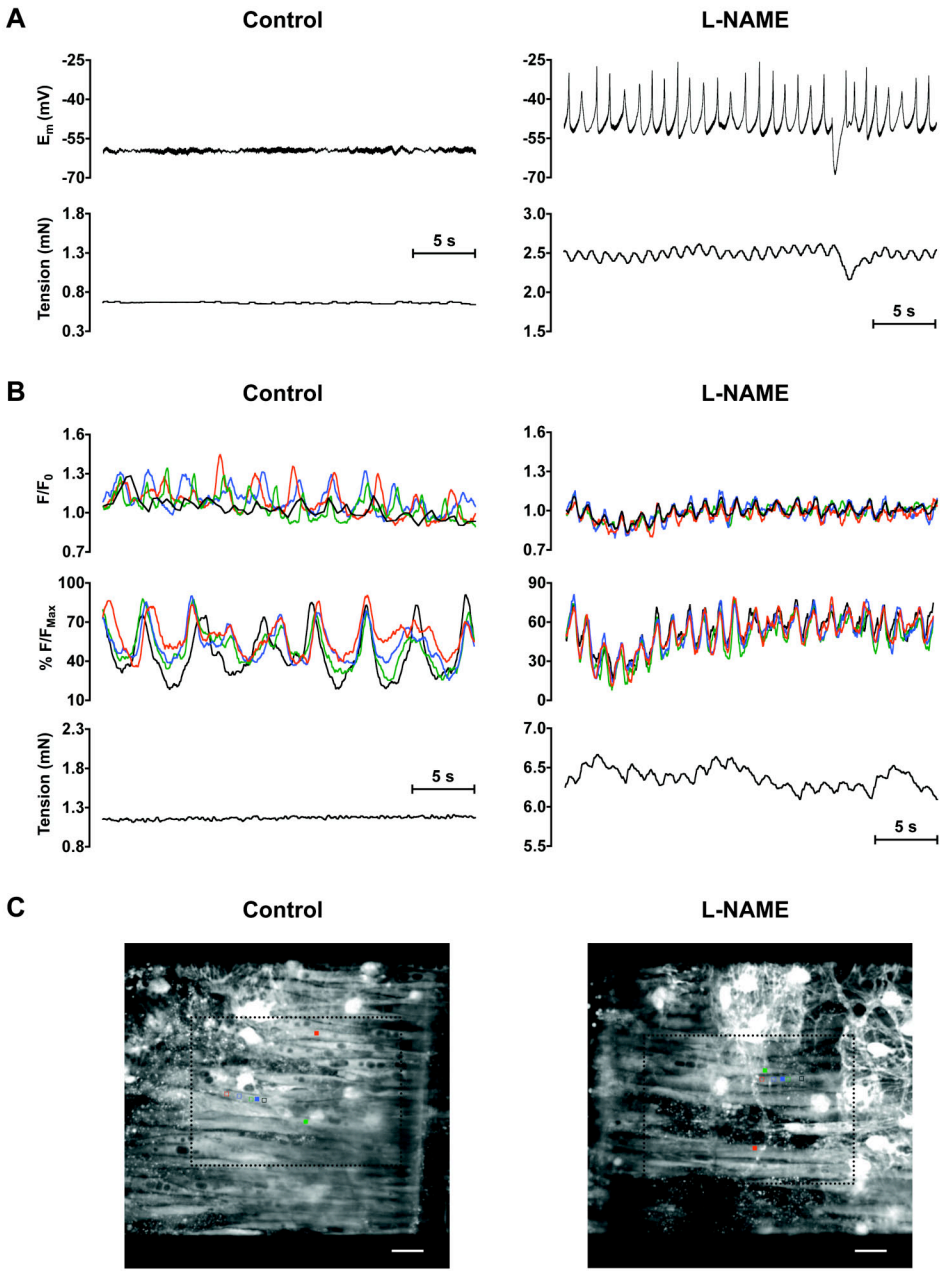

A
Control
L-NAME
E_m (mV)
-25
-40
-55
-70
Tension (mN)
1.8
1.3
0.8
0.3
3.0
2.5
2.0
1.5
5 s
B
Control
L-NAME
F/F0
1.6
1.3
1.0
0.7
% F/FMax
100
70
40
10
90
60
30
0
Tension (mN)
2.3
1.8
1.3
0.8
7.0
6.5
6.0
5.5
5 s
C
Control
L-NAME

Figure 2

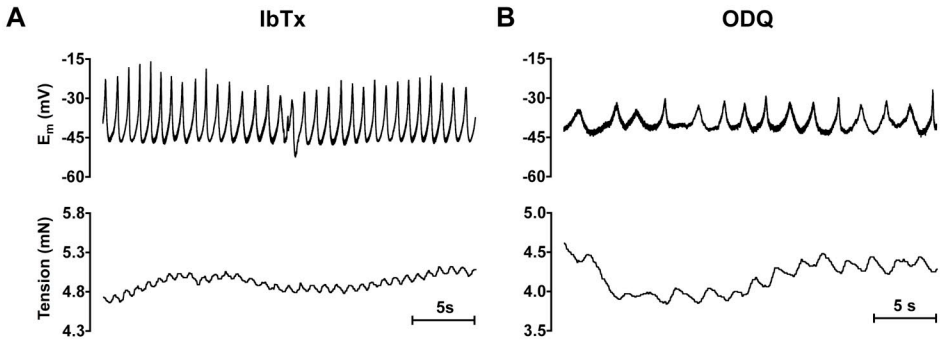

A
IbTx
B
ODQ
-15
-30
-45
-60
E_m (mV)
5.8
5.3
4.8
4.3
Tension (mN)
5s
5.0
4.5
4.0
3.5
5 s

Figure 3

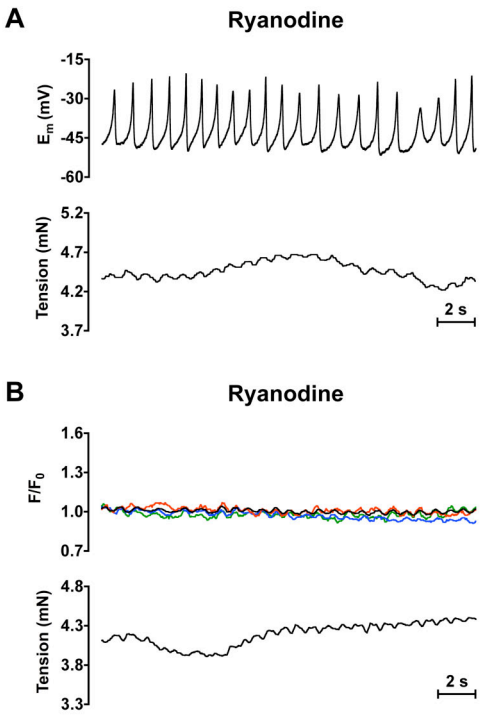

A
Ryanodine
-15
-30
-45
-60
E_m (mV)
5.2
4.7
4.2
3.7
Tension (mN)
2 s
B
Ryanodine
1.6
1.3
1.0
0.7
F/F0
4.8
4.3
3.8
3.3
Tension (mN)
2 s

Figure 4

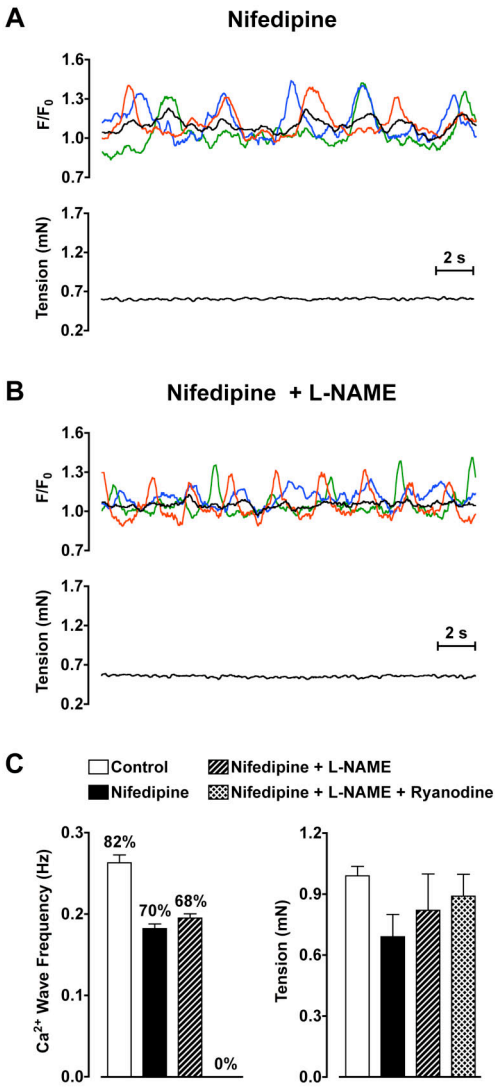

A
Nifedipine
F/F0
1.6
1.3
1.0
0.7
Tension (mN)
1.7
1.2
0.7
0.2
2 s
B
Nifedipine + L-NAME
F/F0
1.6
1.3
1.0
0.7
Tension (mN)
1.7
1.2
0.7
0.2
2 s
C
Control
Nifedipine
Nifedipine + L-NAME
Nifedipine + L-NAME + Ryanodine
Ca2+ Wave Frequency (Hz)
0.3
0.2
0.1
0.0
82%
70%
68%
0%
Tension (mN)
1.2
0.9
0.6
0.3
0.0

Figure 5

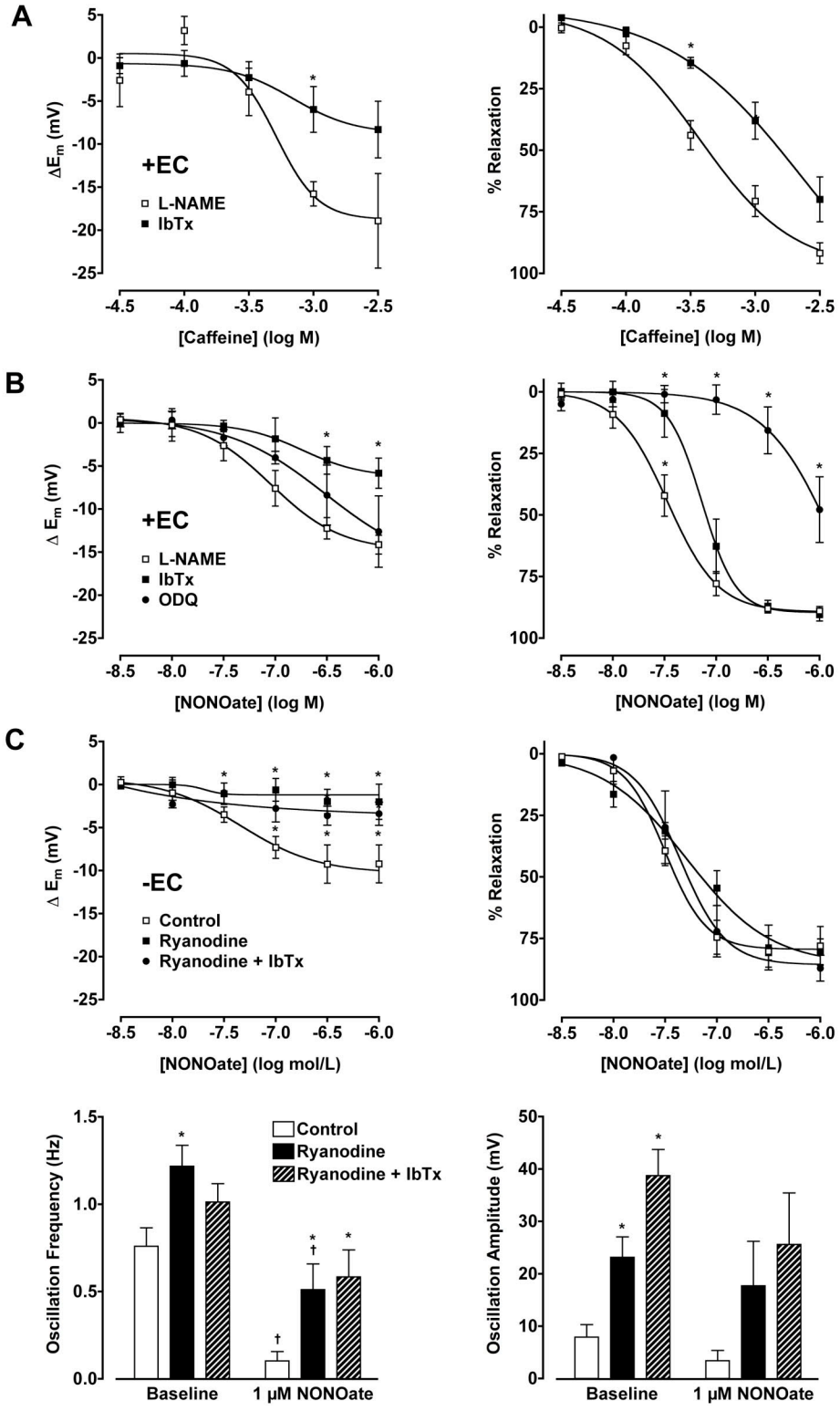

A
ΔEm (mV)
+EC
L-NAME
IbTx
[Caffeine] (log M)
% Relaxation
[Caffeine] (log M)
B
Δ Em (mV)
+EC
L-NAME
IbTx
ODQ
[NONOate] (log M)
% Relaxation
[NONOate] (log M)
C
Δ Em (mV)
-EC
Control
Ryanodine
Ryanodine + IbTx
[NONOate] (log mol/L)
% Relaxation
[NONOate] (log mol/L)
Oscillation Frequency (Hz)
Control
Ryanodine
Ryanodine + IbTx
Baseline
1 μM NONOate
Oscillation Amplitude (mV)
Baseline
1 μM NONOate

Figure 6

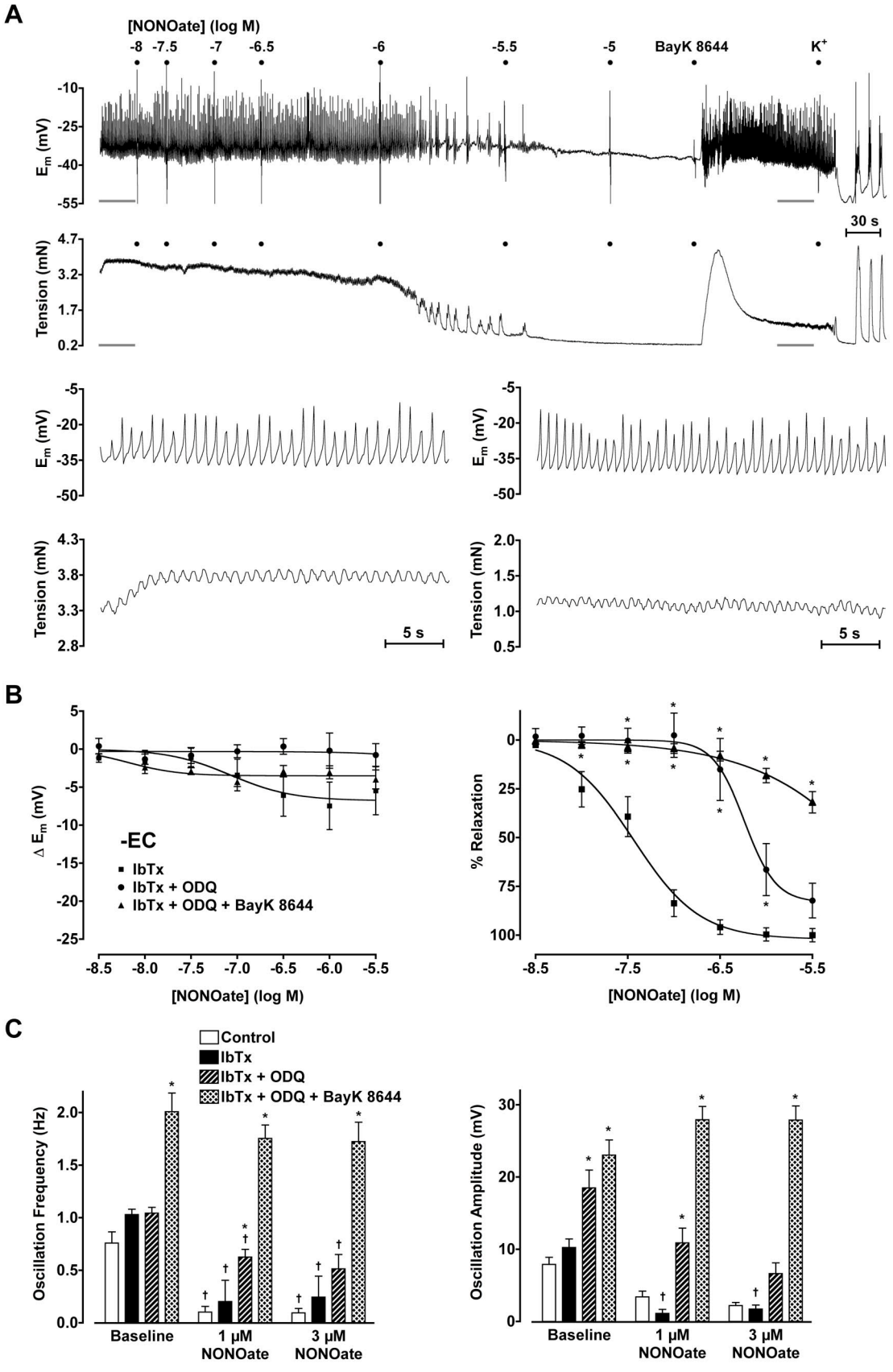

A
[NONOate] (log M)
-8 -7.5 -7 -6.5 -6 -5.5 -5 BayK 8644 K+
Em (mV)
Tension (mN)
30 s
5 s
B
ΔEm (mV)
-EC
IbTx
IbTx + ODQ
IbTx + ODQ + BayK 8644
[NONOate] (log M)
% Relaxation
C
Control
IbTx
IbTx + ODQ
IbTx + ODQ + BayK 8644
Oscillation Frequency (Hz)
Oscillation Amplitude (mV)
Baseline
1 μM NONOate
3 μM NONOate

Figure 7

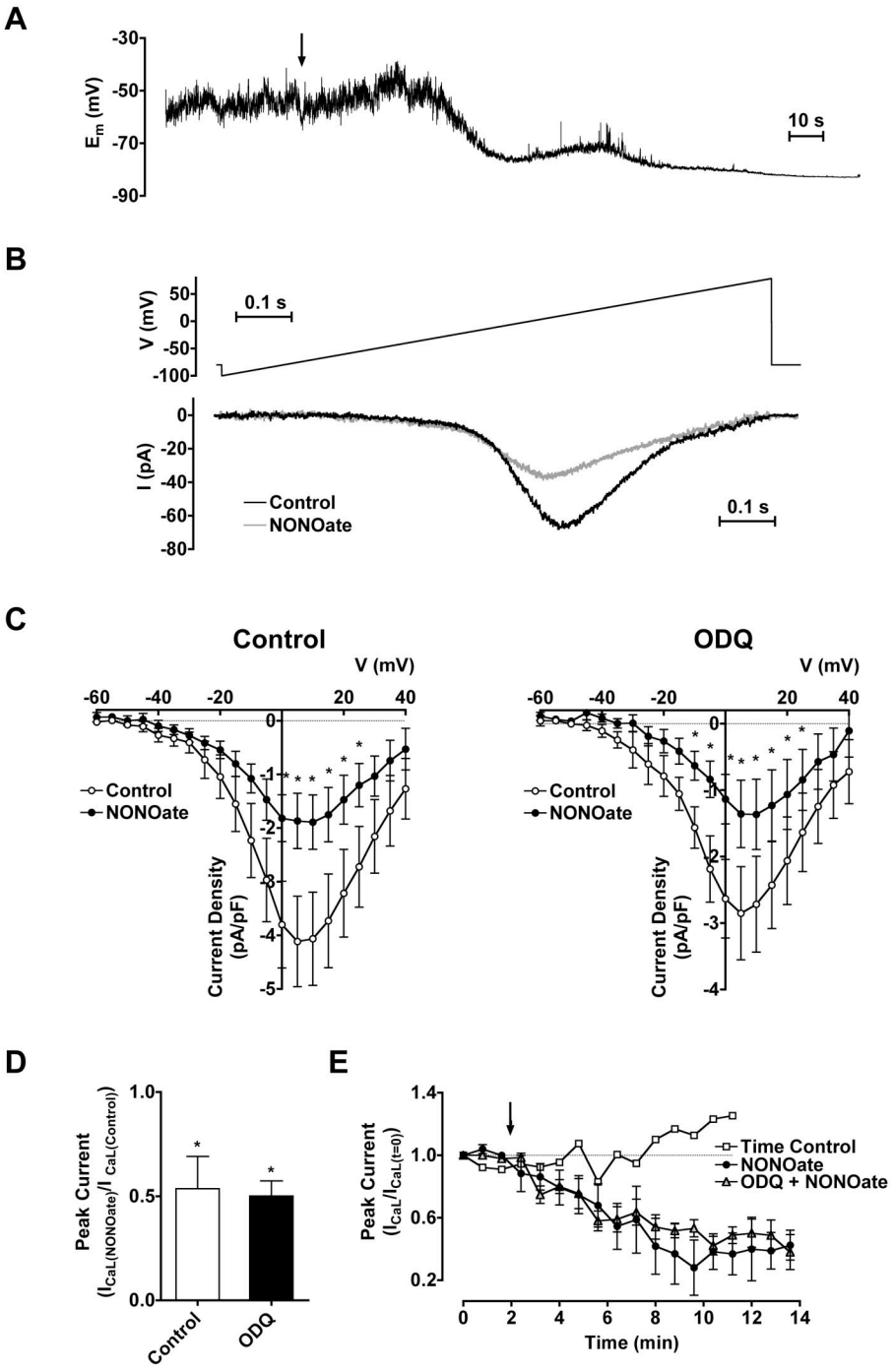
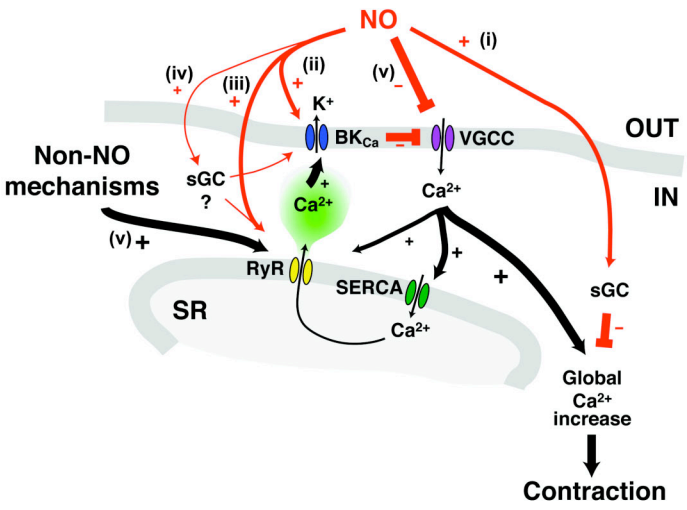

A
-30
-50
-70
-90
E_m (mV)
10 s
B
50
0
-50
-100
V (mV)
0.1 s
0
-20
-40
-60
-80
I (pA)
Control
NONOate
0.1 s
C
Control
ODQ
V (mV)
-60 -40 -20 20 40
0
-1
-2
-3
-4
-5
Control
NONOate
Current Density (pA/pF)
D
Peak Current ($I_{CaL(NONOate)}$/ $I_{CaL(Control)}$)
1.0
0.5
0.0
Control
ODQ
E
Peak Current (I_{CaL}/$I_{CaL(t=0)}$)
1.4
1.0
0.6
0.2
0 2 4 6 8 10 12 14
Time (min)
Time Control
NONOate
ODQ + NONOate

Figure 8


NO
+ (i)
(ii)
+
(iii)
+
(iv)
+
(v)
–
K+
BKCa
VGCC
OUT
IN
Non-NO
mechanisms
sGC
?
Ca2+
Ca2+
(v) +
RyR
SERCA
SR
Ca2+
sGC
Global
Ca2+
increase
Contraction